Novel Brain Arteriovenous Malformation Mouse Models for Type 1 Hereditary Hemorrhagic Telangiectasia

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Abstract

Endoglin (ENG) is a causative gene of type 1 hereditary hemorrhagic telangiectasia (HHT1). HHT1 patients have a higher prevalence of brain arteriovenous malformation (AVM) than the general population and patients with other HHT subtypes. The pathogenesis of brain AVM in HHT1 patients is currently unknown and no specific medical therapy is available to treat patients. Proper animal models are crucial for identifying the underlying mechanisms for brain AVM development and for testing new therapies. However, creating HHT1 brain AVM models has been quite challenging because of difficulties related to deleting Eng-floxed sequence in Eng2fl/2fl mice. To create an HHT1 brain AVM mouse model, we used several Cre transgenic mouse lines to delete Eng in different cell-types in Eng2fl/2fl mice: R26CreER (all cell types after tamoxifen treatment), SM22α-Cre (smooth muscle and endothelial cell) and LysM-Cre (lysozyme M-positive macrophage). An adeno-associated viral vector expressing vascular endothelial growth factor (AAV-VEGF) was injected into the brain to induce focal angiogenesis. We found that SM22α-Cre-mediated Eng deletion in the embryo caused AVMs in the postnatal brain, spinal cord, and intestines. Induction of Eng deletion in adult mice using R26CreER plus local VEGF stimulation induced the brain AVM phenotype. In both models, Eng-null endothelial cells were detected in the brain AVM lesions, and formed mosaicism with wildtype endothelial cells. However, LysM-Cre-mediated Eng deletion in the embryo did not cause AVM in the postnatal brain even after VEGF stimulation. In this study, we report two novel HHT1 brain AVM models that mimic many phenotypes of human brain AVM and can thus be used for studying brain AVM pathogenesis and testing new therapies. Further, our data indicate that macrophage Eng deletion is insufficient and that endothelial Eng homozygous deletion is required for HHT1 brain AVM development.

Introduction

Hereditary hemorrhagic telangiectasia (HHT), also known as Osler-Weber-Rendu (OWR) syndrome, is the first identified human disease caused by defects in a transforming growth factor-β (TGF-β) superfamily receptor [1]. It is an autosomal dominant genetic disorder of the blood vessels that manifests epistaxis, mucocutaneous telangiectases, and arteriovenous malformations (AVMs) in multiple organs including the brain, lung, liver, gastrointestinal tract, and spinal cord [2]. Most of HHT cases are categorized into either HHT type 1 (HHT1) caused by mutations in endoglin (ENG), a TGF-β type III auxiliary receptor mapped on chromosome 9 [1], or HHT type 2 (HHT2) caused by mutations in activin receptor-like kinase 1 (ACVRL1; ALK1), a TGF-β type I serine-threonine kinase receptor located in chromosome 12 [3]. The prevalence of brain AVM in HHT1 patients is 1000-fold higher, and in HHT2, 100-fold higher, than the prevalence in the general population (10/100,000) [4].

Brain AVM is a tangle of abnormal vessels called nidi, where blood directly shunts from arteries into veins without passing the capillary bed. These abnormal vessels tend to rupture, causing spontaneous and recurrent hemorrhages in the brain, especially in children and young adults [5]. The life-threatening intracranial hemorrhage (ICH) risk rate in HHT patients ranges from 1.4% to 2% per year [6]. Currently available therapies, including microsurgery, embolization and/or radiosurgery, are invasive and associated with considerable side effects [7,8]. No specific medical therapy is available for brain AVM patients.

The pathogenesis of HHT brain AVMs remains largely unknown. A reliable animal model is crucial for studying disease mechanisms and testing new therapies. We and others have reported HHT2 developmental [9,10] and adult onset brain AVM phenotypes [11] in Alk1-conditional knockout mice. No compelling HHT1 brain AVM model has been reported.

We have previously shown that microscopic (capillary level) cerebrovascular dysplasia can be induced by focal VEGF stimulation in adult Eng-haploinsufficient (Eng+/−) mice [12]. Arteriovenous (A-V) shunting has not been identified in this model. To create an adult onset HHT1 brain AVM model that closely resembles human disease, we attempted using the same...
strategy that we previously used to develop an HHT2 model: stereotactic injection of a cre-expressing adenoviral vector (Ad-Cre) [11] and an adenovirus-associated viral vector expressing vascular endothelial growth factor (AAV-VEGF). However, Ad-Cre-mediated Eng deletion in the brain of Eng<sup>2fl/2fl</sup> mice was very limited, in only about 1% of cells [13]. Only a few macroscopic level dysplasia vessels developed. The objective of this study is to establish HHT1 brain AVM mouse models that closely mimic the human brain AVM phenotype. The secondary objective is to explore Eng deletion in which cell-type is required for brain AVM development.

We used several cre transgenic lines in this study to achieve effective Eng deletion in Eng<sup>2fl/2fl</sup> mice. Striking brain AVM phenotypes have been developed in Eng<sup>2fl/2fl;SM22α-Cre</sup> mice spontaneously and Eng<sup>2fl/2fl;R26<sup>Cre</sup></sup> mice after tamoxifen (TM) treatment and focal VEGF stimulation. The AVM phenotypes in these models mimic many characteristics of human brain AVM, such as arteriovenous shunting, macrophage infiltration, and microhemorrhage. Although effective Eng deletion in macrophages was achieved, brain AVMs did not develop in Eng<sup>2fl/2fl;Ly5M<sup>Cre</sup></sup> mice after VEGF stimulation, indicating that deletion of Eng in macrophages is not sufficient to cause AVM formation. However, homozygous Eng deletion in endothelial cells might be required for brain AVM development, because Eng-null endothelial cells were detected in AVM vessels in both Eng<sup>2fl/2fl;SM22α-Cre</sup> and Eng<sup>2fl/2fl;R26<sup>Cre</sup></sup> mice.

**Materials and Methods**

**Ethics statement**

The protocol and experimental procedures for using laboratory animals were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of California, San Francisco (UCSF). Animal husbandry was provided by the staff of the IACUC of UCSF, under the guidance of supervisors who are certified Animal Technologists, and by the staff of the Animal Core Facility. Veterinary care was provided by IACUC faculty members and veterinary residents located on the San Francisco General Hospital campus. All mice were housed in a pathogen-free area in 421×316 cm<sup>2</sup> cages with 12 hours light/dark cycle.

**Animals**

To establish various Eng-conditioned knockout mouse lines, Eng<sup>2fl/2fl</sup> (exons 5 and 6 flanked by loxP sites) [14] mice were crossbred with R26<sup>CreER</sup>, SM22α-Cre, and Ly5M-Cre [15] mice (Jackson Laboratory, Bar Harbor, Maine).

**Viral vector stereotactic injection and TM intraperitoneal administration**

AAV-VEGF or AAV-LacZ (vector control) [16] was injected into the brain of 8 to 10-week-old Eng<sup>2fl/2fl;R26<sup>CreER</sup></sup> and Eng<sup>2fl/2fl;Ly5M-Cre</sup> mice (6 mice per group), as previously described [17]. Briefly, mice were anesthetized with isoflurane inhalation and positioned in a stereotactic frame (David Kopf Instruments). A hole was made in the pericranium, 1 mm in diameter and 1 mm deep. Brain samples were harvested and frozen in dry ice, then sectioned into 20 μm sections. The location of AVM lesions was first identified through staining of every 10 sections with lectin (1:200; Vector Laboratories). Prussian blue staining was performed according to the protocol provided by the company (Iron Stain Kit; Sigma-Aldrich).

**Monocytes/macrophage isolation and culture**

Monocytes/macrophages were isolated and cultured as previously described [17]. Briefly, bone marrow was collected from tibias and femurs of 8-week-old Eng<sup>2fl/2fl</sup> and Eng<sup>2fl/2fl;Ly5M-Cre</sup> mice and cultured in a macrophage-enriched medium containing mouse macrophage-colony stimulating factor (M-CSF, 7.5 ng/ml; Akron Biotech) and 10% fetal bovine serum (FBS) for 7 days.

**Real-time quantitative genomic DNA PCR analysis**

DNA was isolated from the brain of 3 control Eng<sup>2fl/2fl</sup> and 3 TM-treated Eng<sup>2fl/2fl;R26<sup>CreER</sup></sup> mice, and macrophages were isolated from 3 Eng<sup>2fl/2fl</sup> and 3 Eng<sup>2fl/2fl;Ly5M-Cre</sup> mice. Recombination of the Eng 2fl (floxed) allele was quantified using Mx3000P QPCR System (Agilent Technologies). The gene deletion efficiency was calculated as the amount of Eng 2fl in experimental samples/Eng<sup>2fl</sup> levels in controls times 100. The primers used for real-time qPCR are listed in Table S1.

**Statistical analysis**

Data are represented as mean ± SD. Student’s t-test was used to determine a statistical significance between groups. A p value of < 0.05 was considered statistically significant. The survival curve...
of Eng2fl/2fl;SM22α-Cre mice was made and the difference between male and female mice was analyzed using Prism 6 software.

**Results**

**Developmental onset of brain AVM in Eng2fl/2fl;SM22α-Cre mice**

The SM22α-Cre transgenic mouse induced target gene deletion in vascular smooth muscle cells during the embryonic developmental stage. In addition to vascular smooth muscle cells, SM22α-Cre transgene has been shown to mediate gene deletion in endothelial cells and some other cell-types [10]. To test if SM22α-Cre-mediated Eng deletion in the embryo leads to postnatal brain AVM, we crossed Eng2fl/2fl mice with SM22α-Cre mice. Three different mouse groups were obtained: Eng2fl/2fl, Engfl/+;SM22α-Cre, and Engfl/fl;SM22α-Cre. The AVM phenotype (greatly enlarged and tortuous vessels) was detected in the brain of 90% (18 out of 20) of 5-week-old Eng2fl/2fl;SM22α-Cre mice that had Eng homozygous deletion in Cre-expressing cells (Figure 1C and 1F). More than 80% of the mice had one lesion, while the remaining 20% had 2–3 lesions. No AVM phenotype was observed in the brain of Engfl/2fl littermates that did not have Cre transgene (wildtype) (Figure 1A and 1D) and Engfl/+;SM22α-Cre mice that had one Eng allele deletion (heterozygous, Figure 1B and 1E).

AVMs were also found in the spinal cord (Figure S2C) and intestine (Figure S2F) of Eng2fl/2fl;SM22α-Cre mice, but not in Engfl/2fl;Engfl/fl;SM22α-Cre mice (Figure S2A, S2B, S2D, and S2E). The brain lesions varied in size, location (Figure S3A and S3B), and number. There was late dye found in the veins (indicative of A-V shunting), and hemorrhage in some brain and spinal cord lesions (Figure S3C and S3D). More than 50% of the Eng2fl/2fl;SM22α-Cre mice died before 6 weeks of age (Figure 1G).

The survival rates were similar in males and females (P = 0.47).

**Adult onset brain AVM in Eng2fl/2fl;R26CreER mice after TM treatment and focal angiogenic stimulation**

To induce the adult onset HHT1 brain AVM phenotype, we utilized the R26CreER transgenic mouse in which Cre expression is activated by TM treatment [9] to conditionally delete the Eng gene. Eng deletion in adult Eng2fl/2fl;R26CreER mice was induced by intraperitoneal injection of TM for 3 consecutive days (Figure 2A). More than 80% of the mice had one lesion, while the remaining 20% had 2–3 lesions. No AVM phenotype was observed in the brain of Eng2fl/2fl;R26CreER mice (Figure S2A, S2B, S2D, and S2E). The brain lesions varied in size, location (Figure S3A and S3B), and number. There was late dye found in the veins (indicative of A-V shunting), and hemorrhage in some brain and spinal cord lesions (Figure S3C and S3D). More than 50% of the Eng2fl/2fl;SM22α-Cre mice died before 6 weeks of age (Figure 1G). The survival rates were similar in males and females (P = 0.47).

No AVM found in Eng2fl/2fl;LysMCre mice

We have previously shown that bone marrow-derived macrophages home to the brain angiogenic foci [10]. Eng+/− mice and WT mice transplanted with Eng+− bone marrow developed a similar degree of dysplasia after VEGF stimulation [11]. These data suggest that bone marrow-derived macrophages may play a role in brain AVM development. To test if Eng deletion in macrophages is sufficient to induce brain AVMs, we utilized the LysMCre mouse [13] that specifically mediates target gene recombination in lysozyme M-positive macrophages. Eng2fl/2fl;LysMCre mice are born with the expected Mendelian ratio and develop normally into the adult stage. Injection of AAV-VEGF into the basal ganglia of 10–10-week-old Eng2fl/2fl;LysMCre mice did not induce AVM phenotype in the brain (Figure 3). Similarly, no AVM phenotype was observed around the ear wound (Figure S4C) and intestine (Figure S4D). Real-time genomic DNA qPCR analysis confirmed that more than 90% Eng was deleted in macrophages of Eng2fl/2fl;LysMCre mice (Figure S5), indicating that the lack of AVM phenotype was not due to inefficient gene deletion. Thus, these data suggest that Eng deletion in lysozyme M-positive macrophages alone was not sufficient to initiate AVM formation.

Eng-null endothelial cells detected in brain AVM vessels

Dilated and dysmorphic (dysplasia) vessels were found in lesions in stained brain sections of 5-week-old Eng2fl/2fl;SM22α-Cre mice (Figure 4A) and around the brain angiogenic region of TM-treated adult Eng2fl/2fl;R26CreER mice (Figure 4B), but not in the brain angiogenic region of adult Eng2fl/2fl;LysMCre mice (Figure 4C). Endothelial ENG expression was greatly reduced in the lesions of brain sections from Eng2fl/2fl;SM22α-Cre and Eng2fl/2fl;R26CreER mice (Figure 4D and 4E). Endothelial ENG expression in the angiogenic foci of Eng2fl/2fl;LysMCre mice did not change (Figure 4F and 4I). Notably, ENG-null endothelial cells were found in large dysplastic vessels in cerebral lesions in both Eng2fl/2fl;SM22α-Cre (Figure 4G) and Eng2fl/2fl;R26CreER (Figure 5H) mice. Further, similar to the phenotype of the Alk1-deficient model (HHT2 model) [19] and human unruptured brain AVMs [20, 21], macrophages and microhemorrhage were observed around these dysplastic vessels in the brain of Eng2fl/2fl;SM22α-Cre and Eng2fl/2fl;R26CreER mice (Figure 5). Thus, all of the evidence indicates that homozygous deletion of Eng in endothelial cells is necessary for the development of macroscopic AVM-like vessels with arteriovenous (A-V) shunts in the mouse brain.

**Discussion**

In this study, we established two novel HHT1 brain AVM mouse models: one, developmental onset, and the other, adult onset. These models are better suited for mechanistic studies and new drug tests than the existing HHT brain AVM models we and others have developed [9–12]. We have also shown that endothelial homozygous Eng deletion is required for the development of the HHT1 brain AVM phenotype, whereas deletion of Eng in macrophages is insufficient.

As a model for investigating AVM pathogenesis and testing novel therapies, our HHT1 developmental model created in Eng2fl/2fl;SM22α-Cre mice has some advantages compared to the two HHT2 developmental brain AVM models developed in Alk1−/−;L1Cre mice [9] and Alk1−/−;SM22α-Cre mice [10]. The Alk1−/−;L1Cre mice died at postnatal day 5 due to massive intracranial hemorrhage [9]. More than 50% of Alk1−/−;SM22α-Cre mice died before 2 weeks of age [10]. Such a short lifespan limits their use in testing new therapies. In contrast, we did not
observe any lethality in our \( \text{Eng}^{2f/2f} ; \text{SM22} \alpha \text{-Cre} \) model until postnatal week 3. About 30% of the mice died between 3 and 6 weeks due to cerebral and/or gastrointestinal hemorrhage. The penetrance of the brain AVM phenotype was about 90% at 5 weeks of age. Further, there is no other manipulation, such as angiogenic stimulation, needed to initiate brain AVM development in addition to gene deletion. Thus, brain AVM initiation and progression in \( \text{Eng}^{2f/2f} ; \text{SM22} \alpha \text{-Cre} \) mice more closely resemble spontaneously developed human brain AVM, making this model more suitable for studying brain AVM pathogenesis and testing new therapies. Since the lesion size, location, and number in this model are highly variable, identifying the location of the AVM lesion and quantifying the dysplasia index by tissue section would be time consuming and challenging. However, the AVM lesions were only detected in \( \text{Eng}^{2f/2f} ; \text{SM22} \alpha \text{-Cre} \) mice, and not in \( \text{Eng}^{2f/2f} \) or \( \text{Eng}^+/2f/ ; \text{SM22} \alpha \text{-Cre} \) mice (Figure 1). We recorded the presence of large AVMs in a whole-mount brain preparation following latex casting in 90% of \( \text{Eng}^{2f/2f} ; \text{SM22} \alpha \text{-Cre} \) mice. This method detected lesions with A–V shunts (latex dye presented in the veins). More than 80% of the mice had one lesion, and the remaining 20%, 2–3 lesions. Thus, comparing the number of mice with lesions in the treated and control groups could be used to evaluate the therapeutic efficacy of new test drugs. In addition, since \( \text{Eng}^{2f/2f} ; \text{SM22} \alpha \text{-Cre} \) mice died at various times (Figure 1G), analysis of the mortality rate could be another means of evaluating a test drug’s effectiveness. More detailed quantification would be extremely time consuming due to the non-specific location of the lesions, which is a limitation of the \( \text{Eng}^{2f/2f} ; \text{SM22} \alpha \text{-Cre} \) model.

The capillary level dysplasia in the brain of \( \text{Eng}^+/2f/ \) mice described previously does not closely resemble the human brain AVM phenotype [12]. The major drawback of our published adult onset brain AVM model is that a Cre-expressing adenoviral vector was used to induce focal \( \text{Alk1} \) deletion [11]. Because the adenoviral vector induces potent local inflammation [22], it limits the use of this model to the study of the role of inflammation in brain AVM phenotype development and progression. We have overcome this limitation in the present model by using the \( \text{R26} \text{CreER} \) transgene to achieve conditional \( \text{Eng} \) deletion. Unlike \( \text{Alk1}^{2f/2f} ; \text{R26} \text{CreER} \) mice that exhibited 100% lethality 2 to 3 weeks after TM-induced gene deletion [9], \( \text{Eng}^{2f/2f} ; \text{R26} \text{CreER} \) mice treated with our selected TM dose survived more than 2 months after the induction of \( \text{Eng} \) deletion. After intra-brain injection of AAV-VEGF, \( \text{Eng}^{2f/2f} ; \text{R26} \text{CreER} \) mice developed the phenotype resembling human brain AVM. AAV causes fewer host inflammatory and immune responses than adenoviral vectors [22]. Therefore, this is a better model for studying the role of inflammation in brain AVM pathogenesis than the model induced by Ad-Cre/AAV-VEGF-mediated focal \( \text{AlkI} \)-deletion and angiogenesis [11,19].

Figure 1. Developmental onset AVM in the postnatal brain of \( \text{Eng}^{2f/2f} ; \text{SM22} \alpha \text{-Cre} \) mice. Representative images of latex dye casting show the cerebrovasculature in the brain of 5-week-old (A) \( \text{Eng}^{2f/2f} \), (B) \( \text{Eng}^{+/2f} ; \text{SM22} \alpha \text{-Cre} \), and (C) \( \text{Eng}^{2f/2f} ; \text{SM22} \alpha \text{-Cre} \) mice. D–F: Enlarged images of dotted boxes shown in A–C after the brain tissue was cleared in organic solvent. The latex-perfused vasculature inside the brain can be observed. Tangled and dilated vessels were detected only in the brain of \( \text{Eng}^{2f/2f} ; \text{SM22} \alpha \text{-Cre} \) mice. Scale bars: 1 mm in A–C and 500 μm in D–F. G: Survival curve of \( \text{Eng}^{2f/2f} ; \text{SM22} \alpha \text{-Cre} \) mice. There was no difference between male (M) and female (F) mice (P = 0.47).
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We detected AVM only in the intestines of $Eng^{2f/2f}$;SM22a-Cre mice, but not in $Eng^{2f/2f}$;R26CreER mice. Interestingly, adult $Alk1^{2f/2f}$;R26CreER mice developed AVMs in their lungs and intestines after TM treatment [9]. Additionally, $Eng^{2f/2f}$;R26CreER mice survived 2 months after TM treatment, whereas all $Alk1^{2f/2f}$;R26CreER mice died within 2 to 3 weeks after TM. We hypothesize that ineffective gene deletion in $Eng^{2f/2f}$;R26CreER was responsible for some of the phenotypic difference between $Eng^{2f/2f}$;R26CreER and $Alk1^{2f/2f}$;R26CreER. We found in our previous study that stereotactic injection of the same dose of Ad-Cre resulted in 16% $Alk1$ deletion in the brain of $Alk1^{2f/2f}$ mice and less than 1% $Eng$ deletion in $Eng^{2f/2f}$ mice [13]. In a future study, we will use a more powerful gene deletion system to test this hypothesis.

The prevailing view regarding AVM manifestation in HHT is that it is caused by haploinsufficiency of one of its causative genes. However, $ENG$ haploinsufficiency in endothelial cells in human HHT lesions appears to be insufficient for AVM development [23]. We found that $Eng$ homozygous deletion in endothelial cells seems to be required for brain AVM formation. Both SM22a-Cre and R26CreER mediate recombination of loxP sites in endothelial cells [9,10]. The fact that the brain AVM phenotype developed only in $Eng^{2f/2f}$;SM22a-Cre and $Eng^{2f/2f}$;R26CreER mice, but not in $Eng^{2f/2f}$;SM22a-Cre and $Eng^{2f/2f}$;R26CreER mice, suggests that $Eng$ homozygous deletion is necessary. These data are consistent with our previous findings that: (1) only capillary level (microscopic level) vascular dysplasia developed in the brain of adult $Eng^{+/2}$ and $Alk1^{+/2}$ mice after intra-brain injection of AAV-VEGF [12]; and (2) $Eng$ homozygous deletion of ~1% endothelial cells in the adult mouse brain was sufficient to induce the macroscopic level of cerebrovascular dysplasia phenotype [13]. Hence, loss of function of the normal allele in the endothelium of HHT patients might be necessary for AVM formation. Many factors contributing to the inactivation or downregulation of the normal allele’s function have been suggested, such as focal second-hit somatic mutations, shedding of $ENG$ from endothelial cells during inflammation [24,25], and reduced endothelial $ENG$ signaling due to increased levels of soluble endoglin (s$ENG$) [26].

In addition, recent studies indicate that bone marrow-derived cells play a crucial role in tissue repair and angiogenesis.
We have shown that Eng$^{+/−}$ mice and WT mice transplanted with Eng$^{+/−}$ bone marrow developed a similar degree of capillary level cerebrovascular dysplasia after VEGF stimulation [17]. These data suggest that bone marrow cells are involved in brain AVM pathogenesis. Macrophage is the major bone marrow-derived cell type detected in the brain angiogenic foci [17,18,28]. To explore the role of macrophages in brain AVM development, we used LysM-Cre transgenic mouse lines to delete Eng in

![Figure 3. AVM phenotype was not detected in the brain angiogenic region of Eng$^{2f/2f}$ LysM-Cre mice.](image)

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Figure 4. ENG-null endothelial cells in dysplastic vessels. Representative images of lectin-stained brain sections from (A) AVM lesion of 5-week-old Eng$^{2f/2f}$;SM22α-Cre, (B) VEGF-induced angiogenic focus of TM-treated adult Eng$^{2f/2f}$;R26CreER, and (C) VEGF-stimulated angiogenic focus of adult Eng$^{2f/2f}$;LysM-Cre mice. ENG expression in (D) Eng$^{2f/2f}$;SM22α-Cre, (E) Eng$^{2f/2f}$;R26CreER, and (F) Eng$^{2f/2f}$;LysM-Cre brain. G-I: Enlarged images of the dotted boxes shown in D-F. Arrows indicate ENG-negative endothelial cells. Scale bars: 100 μm in A–F and 10 μm in G–I.

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We previously found that a systemic reduction in endoglin levels led to a temporal difference in macrophage responses. Eng+/− mice had fewer CD68+ cells in the peri-infarct area at 3 days but more CD68+ cells at 60 days after permanent occlusion of a distal middle cerebral artery [33]. It appears that in the early stages of injury, there are fewer monocytes homing to the injury site. At the late stage when recovery normally occurs, more macrophages persist around abnormal vessels, potentially promoting disease progression. In addition, extravasation of blood content from dysplastic AVM vessels could also attract macrophage infiltration [19,21]. Indeed, macrophages were mostly co-localized with iron deposits around the dysplastic vessels in the two models we present in this manuscript (Figure 5). Further study will be needed to understand how macrophages enter the AVM lesion, and the correlation between macrophage load and patient outcomes.

A limitation of this study is that vascular leakage and macrophage infiltration were not quantified in these models. However, we did observe that these phenotypes were not as severe as in the Alk1-deficient model we previously reported [19]. The potential reasons include: (1) the vascular defect takes longer to develop in Eng-deficient vessels than in Alk1-deficient vessels; and (2) the gene deletion in our previous Alk1 model was induced by Ad-Cre, and as a result, the inflammatory response to adenoviral vector could have resulted in increased macrophage infiltration and more severe vascular leakage [19]. In addition, ineffective gene deletion in Eng2fl/2fl;R26CreER could also be a reason for a milder phenotype. Stereotactic injection of the same dose of Ad-Cre resulted in 16% Alk1 deletion in the brain of Alk12fl/2fl mice and less than 1% Eng deletion in Eng2fl/2fl mice [13]. In the future, we will observe the lesion for a longer period of time, or use a more powerful cre system.

Although many advances have been made in the HHT field, the underlying molecular and cellular mechanisms for AVM formation are still unclear. Our novel mouse brain AVM models would be valuable resources for dissecting brain AVM pathogenesis. Studying more common familial brain AVM cases will also allow us to understand the progression of brain AVM, paving the way towards improving patient care. Further, preclinical studies using these animals will lead to the design of a safer and more effective pharmacological therapy for HHT brain AVM patients, which may also apply to patients with sporadic brain AVM.

**Supporting Information**

Table S1 Primers used for real-time quantitative genomic DNA PCR analysis. Matrix metalloproteinase 9 (Mmp9) was used as an internal quantitative control.

**Figure S1** Conditional Eng deletion in the adult mouse using the R26CreER transgenic mouse. A: Experimental design. TM was injected to Eng2fl/2fl;R26CreER mice i.p. once per day for 5 consecutive days. AAV-VEGF was injected into the right basal ganglia at the time when the first dose of TM was given. Samples were collected for phenotype analysis 8 weeks after the TM and AAV-VEGF injection. B: Quantification of WT Eng (2fl) allele in the genomic DNA isolated from the brain 8 weeks after the first tamoxifen (TM) injection. n = 3 per group.

**Figure S2** SM22α-Cre-driven Eng deletion resulted in AVMs in the postnatal spinal cord and intestine. AVM
phenotypes in the (C) spinal cord and (F) intestine of \textit{Eng}^{2fl/2fl}; SM22α-Cre mice, but not in those of control mice (A, B, D, and E). Arteries (a) and veins (v) are shown in dark and light blue, respectively, in the intestine of \textit{Eng}^{2fl/2fl}; SM22α-Cre mice. Scale bars: 1 mm. (TIF)

Figure S3 AVMs and microhemorrhages found in the brain and spinal cord of 5-week-old \textit{Eng}^{2fl/2fl}; SM22α-Cre mice. Examples of (A) superficially and (B) deeply located brain AVMs. Hemorrhages (arrows) detected in some lesions of the (C) brain and (D) spinal cord. a: Artery, v: Vein. Scale bars: 1 mm. (TIF)

Figure S4 \textit{R26\textsubscript{Cre}}-mediated conditional \textit{Eng} deletion induced de novo skin AVMs around the ear wound. A: AVM vessels around the ear wound (*) of tamoxifen-treated \textit{Eng}^{2fl/2fl}; \textit{R26\textsubscript{Cre}} mice. B: No arteriovenous (A–V) shunts in the intestine of \textit{Eng}^{2fl/2fl}; \textit{R26\textsubscript{Cre}} mice 8 weeks after tamoxifen treatment. No abnormal vascular phenotype observed around the ear wound. (TIF)

References